

Real-Time Polymerase Chain Reaction Assays for Rickettsial Diseases

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ABSTRACT

Introduction/Rationale: Arthropod-borne rickettsial diseases are found worldwide and have been the cause of significant amounts of suffering, disability and fatalities among both military and civilian populations throughout history. Because of the similarity to many infectious diseases in signs and symptoms rickettsial diseases are difficult to diagnose clinically. Moreover, due to the time it takes for antibodies to develop and the low concentration of rickettsial agents in the blood stream the diseases are also difficult to diagnose by laboratory methods. For that reason we have developed real-time PCR assays to detect rickettsial disease agents both at the genus and the species level.

Methods: Real-time PCR assays were developed to identify: 1) pathogenic **Rickettsia**; 2) **Rickettsia prowazekii** and **R. rickettsii**, the etiological agents for epidemic typhus and Rocky Mountain spotted fever (RMSF) and potential BW agents; **R. typhi** and **R. felis** the flea-borne typhus disease agents and **Orientia** (formerly **Rickettsia**) **tsutsugamushi** the scrub typhus agent. The assays utilize molecular beacon probes, which fluoresce when they encounter the target DNA sequence. By manipulating the annealing temperature, and magnesium, probe and primer concentrations of the assays, the optimal conditions were determined. A panel of 22 strains of rickettsiae, 20 strains of orientiae and 19 species of non-rickettsial agents were used to determine the specificity of the assays. Plasmids encoding the target sequences were used to calculate the sensitivity of the assays.

Results: The rickettsial real-time assays were found to be specific: **Rickettsia** assay only was positive for rickettsial and not other bacterial nucleic acid; **R. prowazekii** assay detected four strains of **R. prowazekii**, but not **R. typhi**, any of eight spotted fever rickettsiae, **O. tsutsugamushi** or 11 non-rickettsial bacteria. **R. rickettsii** assay detected two strains of **R. rickettsii** but not any other spotted fever or typhus rickettsiae. **R. typhi** and **R. felis** only reacted to the DNA extracted from **R. typhi** and **R. felis**. **O. tsutsugamushi** assay detected 20 strains of **Orientia** but did not react to 17 strains of **Rickettsia** or 19 species of unrelated bacteria. The sensitivity of the assays was quite good with detection routinely at the level of 3 to 10 copies per reaction.

Conclusion: These real-time PCR assays were found to be capable of detecting rickettsial diseases agents quickly and with great sensitivity and specificity.

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1.0 INTRODUCTION

Arthropod-borne rickettsial diseases are found worldwide and have been the cause of significant amounts of suffering, disability and fatalities among both military and civilian populations throughout history. Because of the similarity to many infectious diseases in signs and symptoms rickettsial diseases are difficult to diagnose clinically. Moreover, due to the time it takes for antibodies to develop and the low concentration of rickettsial agents in the blood stream the diseases are also difficult to diagnose by laboratory methods. For that reason we have developed the very sensitive and specific real-time PCR assays to detect rickettsial disease agents.

1.1 Rickettsial Diseases

Rickettsial diseases are caused by infection with obligate intracellular Gram negative bacteria transmitted by arthropod vectors (see Table I). Because of their obligate host cell requirement these rickettsial agents may not be grown up on artificial media, but must be grown in living cells such as those found in tissue culture, fertilized eggs or laboratory animals. Rickettsial diseases are similar in that they commonly produce high fever and severe headache in patients. Rash may be seen, especially in typhus, some of the spotted fever diseases and scrub typhus. Because the signs and symptoms of rickettsial diseases are not very distinctive they are often confused with many other infectious diseases (e.g. malaria, dengue and typhoid fever). The pathology of rickettsial diseases is associated with the infection of endothelial cells by the rickettsial agents, producing disease in potentially all organs and tissues of the body. The rickettsial diseases are normally self-limiting though they may be quite severe and life threatening illnesses. Antibiotics, such as one of the tetracyclines and chloramphenicol generally produce dramatic recuperative effects seen within 24-48 hours. However, antibiotic resistance has recently been reported, so new treatment modalities and/or vaccines need to be discovered (Strickman 1995, Watt; Corwin 1999).

1.2 Military importance of Rickettsial Diseases:

Rickettsial diseases have played havoc on military operations throughout history (Rapmund, Kelly DJ 2002). The particular diseases and some of the outcomes of rickettsial diseases on military operations are given below.

1.2.1 Epidemic typhus

Epidemic typhus is believed to be the “plague” that devastated Athens during the Peloponnesian War, and decimated Napoleon’s “Grande Armee” of 500,000 men that crossed the Nieman River into Poland to attack Russia in 1812. The Grande Armee returned to Poland in December with only 40,000 men. In addition to devastating military units, epidemic typhus commonly inflicts high morbidity and mortality upon poor, displaced and institutionalized populations (Raoult 1997, Raoult 1998, Tarasevich). During 1918-22 in Russia it is estimated that 2-3 million people (military and civilians during WWI and the Russia civil wars) died of typhus (Patterson). More recently (1997) in Burundi 45,558 typhus cases were diagnosed among the inhabitants during their country’s civil war (Raoult 1998). Because of epidemic typhus debilitating effects on military and civilian populations, it has been described as a potential BW agent (Eitzen). Moreover, due to its stability in dried feces of human body lice, its infection of humans by inhalation of aerosols, and the fact that just one organism can cause disease, *R. prowazekii* is considered a potential biological threat to be used by terrorists and rogue countries. This is more a concern now with the ability to produce in the laboratory antibiotic resistant strains, for which there is currently no FDA approved vaccine available to protect against (Kelly 2002).

1.2.2 Murine typhus

Murine (endemic) typhus is commonly found throughout the world (Azad). However, it was not until the early 1900’s that murine typhus was determined to be a separate disease from epidemic typhus and therefore its impact on military operations was unknown through WWII. Since then, murine typhus has been found to

have been a major cause of fevers of unknown origin (FUO) during the Vietnam War, especially among US soldiers stationed in bases and cantonments (Azad). Because of its worldwide distribution, murine typhus is a potential threat to military deployments everywhere (Miller, Corwin 1997, Raoult 1998).

1.2.3 Spotted fever

The spotted fevers group (SFG) include the agents that cause Rocky Mountain spotted fever (RMSF), Mediterranean spotted fever, African tick bite fever, Queensland tick typhus, and 10 other pathogenic and 20 other non-pathogenic rickettsiae. SFG rickettsiae have not caused major epidemics among military personnel similar to those associated with epidemic typhus. However in recent years, they have caused some significant outbreaks among U.S. military units training in CONUS and OCONUS and in one soldier returning from Somalia (Williams). During 1989, a military unit that trained in Arkansas and Virginia contracted RMSF. The seropositivity rate for those members that went only to Arkansas was 38% and for those that went just to Virginia was 13%. Forty-four percent of these individuals received medical treatment for their illnesses. In 1990, during exercises in Fort Chaffee, Arkansas, 30 individuals (n=1194) seroconverted to RMSF (Yevich). During a field-training mission in Botswana (1992), approximately 30% of 169 US soldiers sought medical attention due to an outbreak of African tick bite fever (Smoak). These outbreaks, the high exposure of military personnel to highly endemic areas and the rising incidence of RMSF at home (Treadwell) suggest that spotted fever diseases are a threat to military personnel training in tick-infested areas (Goddard).

1.2.4 Scrub typhus

Scrub typhus is caused by infection with *Orientia* (formerly *Rickettsia*) *tsutsugamushi*. It occurs in Asia, northern Australia and the western Pacific region, an area in which approximately one million cases occur each year and over one billion people are at risk for contracting the disease. During WWII, scrub typhus was second only to malaria as a leading cause of lost man-days in the Pacific Theater and in some locations feared more than malaria because of a high mortality rate that could not be controlled (Rapmund). During the Vietnam War scrub typhus was a leading cause of FUO, especially among those individuals deployed to jungle/rural areas (Reiley). Most recently scrub typhus has affected US military personnel conducting JTF-FA missions in Indochina, where possible drug resistance exist (Corwin 1999) and among US Marines training at Camp Fuji, Japan (Jiang 2003a). Moreover, drug resistant scrub typhus reported in Northern Thailand (Strickman, Watt) has increased the need to develop an effective vaccine (Kelly 2002).

1.3 Diagnoses of Rickettsial Diseases

Because of the similarity to many infectious diseases in signs and symptoms rickettsial diseases are difficult to diagnose clinically. Therefore, physicians often rely on laboratory tests to aid them in diagnosing rickettsial diseases. However, due to the time it takes for antibodies to develop and the low concentration of rickettsial agents in the blood stream the diseases are also difficult to diagnose by laboratory methods. Some of the current methodologies used in the laboratory include:

1.3.1 Serologic Assays

Serological assays including indirect immunofluorescent assay (IFA), enzyme-linked immunosorbent assays (ELISA) and Western blot analysis that are currently performed for epidemic typhus, murine typhus, spotted fever and scrub typhus using whole cell antigen preparations produced in our biological safety level (BSL)-3 laboratories (Dasch, Halle, Raoult 1989, Richards 1993). Recent development of ELISA and Western blot assays for scrub typhus have been completed utilizing recombinant proteins (e.g. Kp r56, a recombinant protein representing a truncated 56 kDa outer membrane immunodominant antigen from the Karp strain of *O. tsutsugamushi*) (Ching 1998, Ching 2001, Jiang 2003a). The benefits of using recombinant proteins in these assays are the ease and safety in producing the proteins which are very stable as opposed to the whole cell antigen preparation that requires a BSL-3 laboratory, and is very labor intensive and time consuming to produce (Ching 1998).

1.3.2 Molecular biological Assays

Because rickettsial diseases are often misdiagnosed and detectable antibody levels are not seen until after day ten of the illness (Raoult et al 1986) a more rapid means of diagnosis is desired. Nucleic acid based antigen detection assays have been used to successfully identify rickettsiae in clinical, epidemiological, entomological and research specimens. The polymerase chain reaction (PCR) based assays have been used to detect the nucleic acid of rickettsiae responsible for typhus, spotted fever and scrub typhus (Tzianabos, Webb, Carl, Furuya, Kelly 1994).

1.4 Recent Development of Real-time PCR Assays for Rickettsial Diseases

Very recently, a more specific, sensitive, quantitative and faster nucleic acid based antigen detection assay has been developed to detect infectious disease agent nucleic acid. This assay, quantitative real-time PCR (qPCR), has been utilized in our laboratory to develop rickettsial agent specific detection assays. The development, sensitivity and specificity of these assays are described in this presentation.

2. Materials and Methods

The quantitative real-time PCR assays were performed utilizing a master mix prepared containing ddH₂O, pre-mixed reagents OmniMix HS (Cepheid, Sunnyvale, CA), forward and reverse primers, molecular beacon probes, and MgCl₂ (Invitrogen, Carlsbad, CA). Each 25 ml reaction contained: 25 mM of HEPES buffer, 0.2 mM of dNTPs, 5 mM of MgCl₂, 1 ul of template DNA, 1.5 units of Takara Taq DNA polymerase; and 0.3, 0.2, 0.3, 0.5, 0.4, 0.1 and 0.5 uM of forward and reverse primers, and 0.4, 0.3, 0.2, 0.5, 0.4 and 0.2 uM of probe for the specific qPCR, rickettsiae 17 kDa, *R. prowazekii ompB*, *R. typhi ompB*, *R. felis ompB*, *R. rickettsii ompB*, *O. tsutsugamushi* 47 kDa, bacterial 16S rRNA, respectively. The qPCR were performed in a SmartCycler thermocycler (Cepheid, Sunnyvale, CA). The temperatures and cycle parameters included: initial denaturation of 3 minutes at 94°C; and 50 cycles of denaturation (94°C for 5 sec) and annealing/elongation (60°C for 30 sec). CT (crossing the threshold) value, a positive reaction, was identified by the experimental samples producing fluorescence greater than the calculated threshold value based upon background fluorescence measured during amplification or an adjusted value based on its fluorescence strength. “No template controls” produced at the same time and under the same conditions as the experimental positive control samples were consistently negative (did not cross the threshold of background fluorescence). To determine the sensitivity of the real-time PCR assays, serial dilutions of known concentrations of the target sequence in plasmids for each assay, were assessed as previously described (Jiang 2003b, Jiang 2004).

3. Results

3.1 Epidemic typhus qPCR

Identification of *R. prowazekii* by qPCR was accomplished to the level of detection of three copies of target sequence per microliter. In addition, the assay detected all four strains of *R. prowazekii* evaluated (Breinl, Madrid E, Ananiev and Cairo). However, it did not give a false signal for two strains of the closely related *R. typhi* (Wilmington and Museibov), or any of 8 spotted fever rickettsiae or *O. tsutsugamushi*. In addition, the assay did not falsely detect DNA from 11 unrelated bacteria or from host cell material used to propagate certain bacteria (Table II). Thus, this assay is very sensitive (3 copies/ ul) and specific.

3.2 Murine typhus and cat-flea typhus qPCR

Identification *R. typhi* and *R. felis*, members of the typhus group and spotted fever group rickettsiae, respectively, cause disease in humans following the bite of an infected flea. Because these agents have the capability of being transmitted simultaneously, and produce similar diseases, we developed a qPCR assay for each agent based upon different target sequences of the *ompB* gene. Both assays are agent specific and very sensitive. Utilizing the panels of rickettsial and non-rickettsial bacterial DNA panels, each agent (two strains for *R. typhi* Wilmington and Museibov) and a single isolate of *R. felis* were consistently detected by their corresponding assays, but these same assays did not falsely detect the other agent, other rickettsial species or non-rickettsial species nucleic acid preps (Table II). The assays were sensitive enough to detect as low as three copies of the target sequence in 1 ul samples.

3.3 Rocky Mountain spotted fever qPCR

R. rickettsii, the etiologic agent of RMSF was detected utilizing a qPCR assay based upon an agent specific partial sequence of the *ompB* gene. The assay was sensitive enough to detect down to three target sequence copies per microliter. In addition, the assay was specific enough not to detect two strains of *R. rickettsii* R and 364-D, but did not falsely react with nucleic acid from any of the other 8 spotted fever group or the typhus group rickettsiae. It also did not react falsely with *O. tsutsugamushi* or 11 non-rickettsial nucleic acid preparations.

3.4 Scrub typhus qPCR

The detection of *O. tsutsugamushi* by the scrub typhus qPCR assay was able to detect 20 different strains of *O. tsutsugamushi*, but did not produce false positive responses to 17 rickettsial or 19 bacterial nucleic acid preparations. These preparations were evaluated by other nucleic acid detection assays (17 kDa, 16S rRNA genes) to ensure that the negative reactions were due to specificity of the qPCR assay and not because there was a lack of appropriate nucleic acid in the preparations (Table II). The sensitivity for this assay like the others was efficient enough to detect down to three copies of the target sequence.

4.0 Discussion

With the development of the polymerase chain reaction (PCR), where agent-specific nucleic acid can be amplified a million fold, rickettsial DNA can be readily detected with great sensitivity in clinical specimens such as blood and tissue samples as well as in arthropod vectors (Tzianabos, Webb, Carl, Furuya, Kelly 1994, Richards 1997, Williams). This sensitivity has been enhanced by the development of the nested PCR assays, which are performed using two separate amplification steps. In addition to detection, the amplicon can be used for restriction fragment length polymorphism typing or sequence analysis. Quantitative real-time PCR assays are as sensitive as nested PCR assays but have the additional advantages of increase specificity, faster results and quantitative information, useful for clinical monitoring of appropriate response to treatment and prognosis, and for experimental studies.

In this report we describe the development of six qPCR procedures with agent specific fluorescent probes able to detect pathogenic rickettsial agents. These assays were developed initially as standard PCR (sPCR) assays, albeit with resultant amplicons of only about 100 bp. The assays were optimized utilizing DNA extracted from the target organism (e.g. *R. prowazekii* Breinl, *O. tsutsugamushi* Karp, etc) and following the selection of an appropriate probe they were further optimised for use in the real-time PCR assay. The specificity of the assays was subsequently evaluated utilizing a panel of closely and distantly related bacteria. The panels included nucleic acid preparations from 17 strains of *Rickettsia*, 20 strains of *O. tsutsugamushi*, and 19 species of other bacteria (Table II). Production of the panels was described previously (Jiang 2004).

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Each of the six assays evaluated showed the ability to detect specific target selection, with all strains of the target entity producing positive reactions and no positive responses from the unrelated *Rickettsia*, other bacteria nucleic acid samples assessed, or the host cell DNAs in which the obligate intracellular bacteria were cultivated, indicating that the qPCR assays were specific for their target agent. The integrity of the *Rickettsia* and bacterial DNAs were confirmed with a qPCR assay based upon the conserved *Rickettsia* 17 kDa antigen gene and a sPCR assay for the 16S rRNA gene (Jiang 2004).

To determine the sensitivity of the qPCR assays we utilized plasmids containing the target sequences. These included fragment A of the open reading frame of the *ompB* of *R. prowazekii* and *R. typhi*, and the open reading frame sequence of the Kato 47 kDa gene that was ligated into the plasmid VR1012 (Vical) (pWMC-Kt47). The other qPCR assays utilized the amplicons produced by the sPCR to ligate into the Topo plasmid according to the manufacturer's instructions. The plasmids' concentrations were determined by OD reading at 260 nm. Serial ten-fold dilutions of the plasmids in molecular biology grade water were performed resulting in final target concentrations of 10^7 to 10^0 copies/ μ L. The assays consistently detected between 3-10 copies of the target sequence per reaction.

In summary, this paper describes six qPCR assays for detecting rickettsial pathogens that are sensitive (3-10 copies/reaction) and specific. These assays are currently under evaluation for effectiveness in detecting target sequences within arthropod vectors, blood, urine and other background matrices.

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Table I: Rickettsial Diseases, Rickettsial Agents and their Arthropod Vectors

<u>Group</u>	<u>Disease</u>	<u>Agents</u>	<u>Arthropod Vector</u>
Typhus	Epidemic Typhus	<i>Rickettsia prowazekii</i>	Human Body Louse (<i>Pediculus humanus</i>)
	Murine Typhus	<i>Rickettsia typhi</i>	Oriental Rat Flea (<i>Xenopsylla cheopis</i>) & Cat Flea (<i>Ctenocephalides felis</i>)
Spotted Fever	Rocky Mountain Spotted Fever	<i>Rickettsia rickettsii</i>	Tick (<i>Dermacentor variabilis</i>)
	Mediterranean Spotted Fever	<i>Rickettsia conorii</i>	Tick (<i>Rhipicephalus sanguineus</i>)
	African Tick Bite Fever	<i>Rickettsia africae</i>	Tick (<i>Amblyomma</i> sp)
	Queensland Tick Typhus	<i>R. australis</i>	Tick (<i>Ixodes holocyclus</i>)
	Cat Flea Typhus	<i>Rickettsia felis</i>	Cat Flea (<i>Ctenocephalides felis</i>)
	Rickettsialpox	<i>Rickettsia akari</i>	Mouse Mite (<i>Liponyssoides sanhuineus</i>)
Scrub Typhus	Scrub Typhus	<i>Orientia tsutsugamushi</i>	Chigger (Leptotrombidium)

Table II: Rickettsial specific qPCR with panels of rickettsiae and non-rickettsial bacteria nucleic acid preparations

<i>Rickettsia</i> Isolates	17 kDa sPCR	<i>R. prowazekii</i> <i>ompB</i> qPCR	<i>R. typhi</i> <i>ompB</i> qPCR	<i>R. felis</i> <i>ompB</i> qPCR	<i>R. rickettsii</i> <i>ompB</i> qPCR	Orientia tsutsugamushi 47 kDa qPCR	16S rRNA sPCR
<i>R. prowazekii</i> Breinl	+	+	-	-	-	-	ND
<i>R. prowazekii</i> Madrid E	ND	+	ND	ND	ND	ND	ND
<i>R. prowazekii</i> Ananiev	ND	+	-	-	-	ND	ND
<i>R. prowazekii</i> Cairo	ND	+	ND	ND	ND	ND	ND
<i>R. typhi</i> Wilmington	+	ND	+	-	ND	-	ND
<i>R. typhi</i> Museibov	+	-	+	-	-	-	ND
<i>R. bellii</i> G2D	+	ND	ND	ND	ND	-	ND
<i>R. canada</i> MCK-29	ND	ND	-	-	-	ND	ND
<i>R. rickettsii</i> R(2D)	+	ND	ND	ND	+	ND	ND
<i>R. sp.</i> 364-D	+	-	-	-	+	-	ND
<i>R. conorii</i> ITT	+	-	-	-	-	-	ND
<i>R. montana</i> OSU 85-930	+	-	-	-	-	-	ND
<i>R. africae</i> EthSFC84360	+	ND	ND	ND	ND	-	ND
<i>R. sharonii</i> ISTT CW	+	ND			ND	-	ND
<i>R. parkeri</i> Maculatum C (CWPP)	+	-	-	-	-	-	ND
<i>R. slovaca</i> D	+	-	-	-	-	-	ND
<i>R. japonica</i> NT	+	-	-	-	-	-	ND
<i>R. sibirica</i> 3358	+	-	ND	ND	-	-	ND
<i>R. rhipicephali</i> CWPP	+	ND	ND	ND	ND	-	ND
<i>R. honei</i> TT118	+	ND	ND	ND	ND	-	ND
<i>R. akari</i> Str #29	+	-	-	-	-	-	ND
<i>R. felis</i>	+	ND	-	+	-	-	ND

Orientia tsutsugamushi Strains	17 kDa sPCR	<i>R. prowazekii</i> <i>ompB</i> qPCR	<i>R. typhi</i> <i>ompB</i> qPCR	<i>R. felis</i> <i>ompB</i> qPCR	<i>R. rickettsii</i> <i>ompB</i> qPCR	<i>Orientia</i> <i>tsutsugamushi</i> 47 kDa qPCR	16S rRNA sPCR
Karp	-	-	-	-	-	+	ND
Kato						+	
Gilliam						+	
TA678 PP						+	
TA686 PP						+	
TA716 PP						+	
TA763 PP						+	
TH1813						+	
TH1814						+	
TH1817						+	
TH1818						+	
TH1819						+	
TH1823						+	
AFC3						+	
AFPL12						+	
AF245						+	
AF312						+	
AF316						+	
AF338						+	
MAK110						+	

Real-Time Polymerase Chain Reaction Assays for Rickettsial Diseases

Other Bacterial Species	17 kDa qPCR	<i>R. prowazekii</i> ompB qPCR	<i>R. typhi</i> ompB qPCR	<i>R. felis</i> ompB qPCR	<i>R. rickettsii</i> ompB qPCR	Orientia tsutsugamushi 47 kDa qPCR	16S rRNA sPCR
<i>Ehrlichia chaffeensis</i>	ND	ND	ND	ND	ND	-	+
<i>Neorickettsia sennetsu</i>	-	-	-	-	-	-	+
<i>N. risticii</i>	-	-	-	-	-	-	+
<i>Bartonella quintana</i>	-	-	-	-	-	-	+
<i>B. vinsonii</i>	-	-	-	-	-	-	+
<i>Francisella persica</i>	-	-	-	-	-	-	+
<i>Legionella pneumophila</i>	-	-	-	-	-	-	+
<i>L. bozemanii</i>	ND	ND	ND	ND	ND	-	+
<i>L. micdadei</i>	ND	ND	ND	ND	ND	-	+
<i>Proteus mirabilis</i>	-	-	-	-	-	-	+
<i>Escherichia coli</i>	-	-	-	-	-	-	+
<i>Citrobacter freundii</i>	ND	ND	ND	ND	ND	-	+
<i>Shigella flexneri</i>	ND	ND	ND	ND	ND	-	+
<i>Pseudomonas aeruginosa</i>	ND	ND	ND	ND	ND	-	+
<i>Vibrio cholerae</i>	ND	ND	ND	ND	ND	-	+
<i>Aeromonas hydrophila</i>	ND	ND	ND	ND	ND	-	+
<i>Staphylococcus aureus</i>	-	-	-	-	-	-	+
<i>Corynebacterium</i> sp.	-	-	-	-	-	-	+
<i>Salmonella enterica</i>	-	-	-	-	-	-	+